(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 182 546**
A2

D5

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: **85308070.3**

(22) Date of filing: **06.11.85**

(51) Int. Cl.⁴: **G 01 N 33/541**

(30) Priority: **12.11.84 GB 8428487**

(43) Date of publication of application: **28.05.86 Bulletin 86/22**

(84) Designated Contracting States: **BE CH DE FR GB IT LI NL SE**

(71) Applicant: **POLYCLONAL ANTIBODIES LIMITED, 14/15 Newbury Street, London EC1A 7HU (GB)**

(72) Inventor: **Landon, John, 85 West Street, Harrow-on-the-Hill Middlesex (GB)**

(74) Representative: **Wain, Christopher Paul et al, A.A. THORNTON & CO. Northumberland House 303-306 High Holborn, London WC1V 7LE (GB)**

(54) **Immunoassays.**

(57) In the immunoassay of an antigen using the second antibody technique, the second antibody is raised against the Fc component only of the first antibody. This reduces, or reduces the risk of, interference in the primary reaction by the second antibody. In a modified «two-site» immunoassay, a second antibody is used, directed against the Fc component of the unlabelled first antibody, to insolubilise the first antibody. Single reagent immunoassays may be effected using the modified second antibody technique of the invention.

EP 0 182 546 A2

R5

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 182 546**
A3

(12) EUROPEAN PATENT APPLICATION

(21) Application number: **85308070.3**

(22) Date of filing: **06.11.85**

(51) Int. Cl.⁴: **G 01 N 33/541**

(30) Priority: **12.11.84 GB 8428487**

(43) Date of publication of application: **28.05.86 Bulletin 86/22**

(84) Designated Contracting States: **BE CH DE FR GB IT LI NL SE**

(88) Date of deferred publication of search report: **09.09.87 Bulletin 87/37**

(71) Applicant: **POLYCLONAL ANTIBODIES LIMITED, 14/15 Newbury Street, London EC1A 7HU (GB)**

(72) Inventor: **Landon, John, 85 West Street, Harrow-on-the-Hill Middlesex (GB)**

(74) Representative: **Wain, Christopher Paul et al, A.A. THORNTON & CO. Northumberland House 303-306 High Holborn, London WC1V 7LE (GB)**

(54) Immunoassays.

(57) In the immunoassay of an antigen using the second antibody technique, the second antibody is raised against the Fc component only of the first antibody. This reduces, or reduces the risk of, interference in the primary reaction by the second antibody. In a modified "two-site" immunoassay, a second antibody is used, directed against the Fc component of the unlabelled first antibody, to insolubilise the first antibody. Single reagent immunoassays may be effected using the modified second antibody technique of the invention.

EP 0 182 546 A3

European Patent Office

EUROPEAN SEARCH REPORT

0182546

Application number

EP 85 30 8070

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X	US-A-4 481 298 (R.O. CONE et al.) * complete *	1-10	G 01 N 33/541 G 01 N 33/563// G 01 N 33/74
A	US-A-4 298 592 (W.H.T. LIN et al.) * column 1, line 57 - column 2, line 57 *	1,2,5	
A	US-A-4 469 787 (J.W. WOODS et al.) * abstract *	1,2,5	
A,P	US-A-4 548 908 (N. KAMEDA) * abstract *	1	
A,P	US-A-4 522 922 (J. CARRO et al.) * claims *	1	TECHNICAL FIELDS SEARCHED (Int. Cl.4) G 01 N 33/00

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
BERLIN	15-06-1987	GREEN C.H.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO Form 1503 03.82

IMMUNOASSAYS

This invention relates to immunoassays.

Immunoassays depend upon the reversible and non-covalent binding of an antigen (Ag), which is the analyte to be measured by its specific antibodies (Ab), to form a complex (Ag:Ab), in accordance with the reaction:

$$\underset{\text{(free fraction)}}{Ag + Ab} \rightleftharpoons \underset{\text{(bound fraction)}}{Ag:Ab}$$

At equilibrium, some of the free reactants will be combining to form more complex, and some of the complex will be dissociating to give the free reactants.

In order to enable, or improve the accuracy of, quantitation in immunoassays, it is known to use a labelled reagent. Among the labels commonly used are radioisotopes, enzymes and fluorophors.

In many immunoassays, it is necessary to separate the bound fraction from the free fraction once equilibrium has been established. A large number of physicochemical, immunological and other means of separating the antibody-bound and free fractions have been developed. Among these is the so-called second antibody approach. In accordance with this technique, there is included in the reaction mixture, in addition to the antibody (first antibody) directed against the antigen, another antibody (second

In one aspect, the present invention thus provides a method of immunoassay of an antigen wherein the antigen is reacted with a first antibody to form an antigen:antibody complex, and wherein a second antibody is used to bind to the first antibody to form a precipitate therewith, the precipitate being separated out, characterised in that the second antibody is directed only against the Fc component of the first antibody.

The method of the invention is applicable to all second antibody assays, including those using a labelled reagent, in which there is a separation step.

One particular form of immunoassay with which the present invention may be utilised is that known as a "two-site" technique for the assay of large molecules with more than one antigenic determinant. In a conventional "two-site" assay, the sample under assay is first incubated with an excess of an antibody covalently linked to an insoluble support and directed against one antigenic determinant ($SP\text{-}Ab_1$)

$$Ag + \text{excess } SP\text{-}Ab_1 \longrightarrow SP\text{-}Ab_1{:}Ag + Sp\text{-}Ab_1 \qquad \text{----------(i)}$$

After careful washing, excess of a second, isotopically labelled antibody (Ab_2^*) directed against another part of the antigen molecule is added

$$SP\text{-}Ab_1 + SP\text{-}Ab_1{:}Ag + \text{excess } Ab_2^* \longrightarrow$$
$$SP\text{-}Ab_1 + SP\text{-}Ab_1{:}Ag{:}Ab_2^* + Ab_2^* \quad \text{---------- (ii)}$$

Following further washes, the counts (in the case of a radiolabel) present in the solid phase fraction are determined and are related directly to the concentration of antigen present in the samples and standards.

the assay result. The so-called "single reagent" is, of course, a mixture of all the various reaction components required for the assay (excepting the sample or standard). In theory, single reagent label immunoassays should significantly reduce the complexity of label assays, and increase the speed and precision thereof. Also, they should reduce reagent manufacturing costs and also improve the stability of some isotopically labelled antigens.

However, despite these potential advantages, there have been only a very few proposals hitherto for single reagent label immunoassays and, so far as we are aware, none of these has been very satisfactory. For example, one commercially available kit for the radioimmunoassay of thyroxine (T_4) used a single reagent comprising ^{125}I-T_4, mixed with anti-T_4 antiserum and second antibody raised against the entire immunoglobulin G (IgG) of the species used to make the antiserum. However, in conducting the assay, the mixture of sample (or standard) and single reagent had to be incubated for 30 minutes at 56°C, which was not very convenient. Furthermore, the assay gave relatively poor precision (up to 10% coefficients of variation) and high recovery (up to 125%) as compared with a conventional double antibody assay for T_4. In another proposed radioimmunoassay (RIA) for T_4, it was found that the assay was accurate only if the "single reagent" was made up immediately before use. Again, this is not very convenient and somewhat negates the advantages theoretically achievable by "single reagent" procedures.

We have now found, in accordance with a further aspect of the present invention, by using a second antibody directed against the Fc fragment only of the first antibody, "single reagents" can be made up which are stable and reliable in subsequent use in an assay. Thus, the invention includes a single reagent label immunoassay

with fluorescein isothiocynate according to standard methods. Antiserum to HSA was raised in a sheep by standard procedures. Antisera to sheep IgG and to sheep Fc fragment were raised in rabbits by standard procedures. Assay diluent buffer was sodium phosphate (50 mmol/l pH 7.4) containing bovine serum albumin (5 g/l) and sodium azide (1 g/l). Standard solutions of HSA were prepared in diluent buffer.

Experiments were performed using polystyrene test tubes which also served as cuvettes for measurement of fluorescence in a Perkin-Elmer Model 1000M filter fluorimeter (Kamel et al. (1980) Clin. Chem. 26, 1281-1284).

For the fluoroimmunoassays with conventional reagent addition sequence, working tracer reagent was prepared by diluting the fluorescein-labelled HSA to 2.6 mg/l (based on its protein content) in diluent buffer and working antiserum reagent by diluting the anti-HSA serum by 1,430-fold in diluent buffer.

The assay procedure with conventional second-antibody separation was as follows. To 50 µl of standard was added 200 µl of working tracer reagent followed by 100 µl of working antiserum reagent. After incubation for 1 h at room temperature, 50 µl of normal sheep serum (diluted 1,000-fold in diluent buffer) and 50 µl of second antiserum (either anti-IgG or anti-Fc, each diluted 8-fold in diluent buffer) was added. After further incubation for 2 h at room temperature, the assay mixtures were centrifuged, the supernatants aspirated to waste, and the precipitates re-dissolved by the addition of 1.5 ml of a mixture of methanol, water and 2 mol/l sodium hydroxide (50:50:1 parts by volume). The fluorescence of the re-dissolved precipitates (antibody-bound fraction of the labelled HSA) was measured by placing each test tube into the fluorimeter.

The fluoroimmunoassay standard curves obtained

2. Example of a single reagent immunoradiometric assay employing anti-Fc second antibody: liquid-phase two-site assay of human prolactin

Antisera to human prolactin were raised in sheep and rabbits by standard procedures. Specific anti-prolactin immunoglobulins were isolated from the sheep antiserum by immunoaffinity purification and were radioiodinated with ^{125}I by conventional procedures. Antisera to rabbit IgG and to rabbit Fc fragment were raised in sheep by standard procedures. Assay diluent buffer was sodium phosphate (64 mmol/l, pH 7.4) containing polyethylene glycol 6000 (30 g/l), bovine serum albumin (5 g/l), normal rabbit serum (10 ml/l), normal sheep serum (20 ml/l), and thiomersal (100 mg/l). Standard solutions of human prolactin were prepared in horse serum.

The procedure for conventional liquid-phase two-site assay was as follows. To 100 µl of standard was added 100 µl of rabbit anti-prolactin serum (diluted 400-fold in diluent buffer) followed by 100 µl (approx. 100,000 counts/min) of ^{125}I-labelled sheep anti-prolactin immunoglobulins. After incubation for 3 h at room temperature, 100 µl of sheep anti-(rabbit (IgG) serum (appropriately) diluted in 64 mmol/l sodium phosphate buffer, pH 7.4) was added and after incubation for a further 1 h at room temperature the assay mixtures were centrifuged, the supernatants aspirated to waste and the radioactivity in the precipitates counted. The standard curve obtained is shown in Figure 3.

When the above experiment was repeated employing an appropriate dilution of sheep anti-(rabbit Fc) second antiserum in place of the anti-(rabbit IgG) serum, an identical standard curve was obtained.

The experiment of Figure 3 was repeated employing a mixture of the two specific anti-prolactin reagents that had been prepared several days previously. To 100 µl of standard was added 200 µl

conventional procedure - avoiding two pipetting steps and the need for a second incubation, and with all reagent additions completed at one time.

6. A single reagent label immunoassay for an antigen, in which the antigen is mixed with a single reagent mixture and, optionally after a separation step, the label is assayed, characterised in that the single reagent mixture comprises a first antibody against the antigen and a second antibody against the Fc component of the first antibody.

7. An assay according to claim 6, wherein said antigen has at least two antigenic determinants, and wherein said "single reagent" mixture comprises antibody raised in a first species against a determinant of the antigen under assay; labelled antibody raised in a second species against another determinant of the antigen; and antibody raised in said second species against the Fc fragment of the IgG of the first species.

8. An immunoassay according to claim 6 or 7, wherein the label is a radioisotope, enzyme or fluorophore.

9. A "single reagent" mixture for use in the assay of claim 6, which comprises a first antibody against an antigen, and a second antibody against the Fc component of the first antibody.

10. A "single reagent" mixture for use in the assay of claim 7, which comprises antibody of a first animal species against an antigen, labelled antibody of a second animal species against the antigen, and antibody raised in said first species against the Fc component of the IgG of the first species.

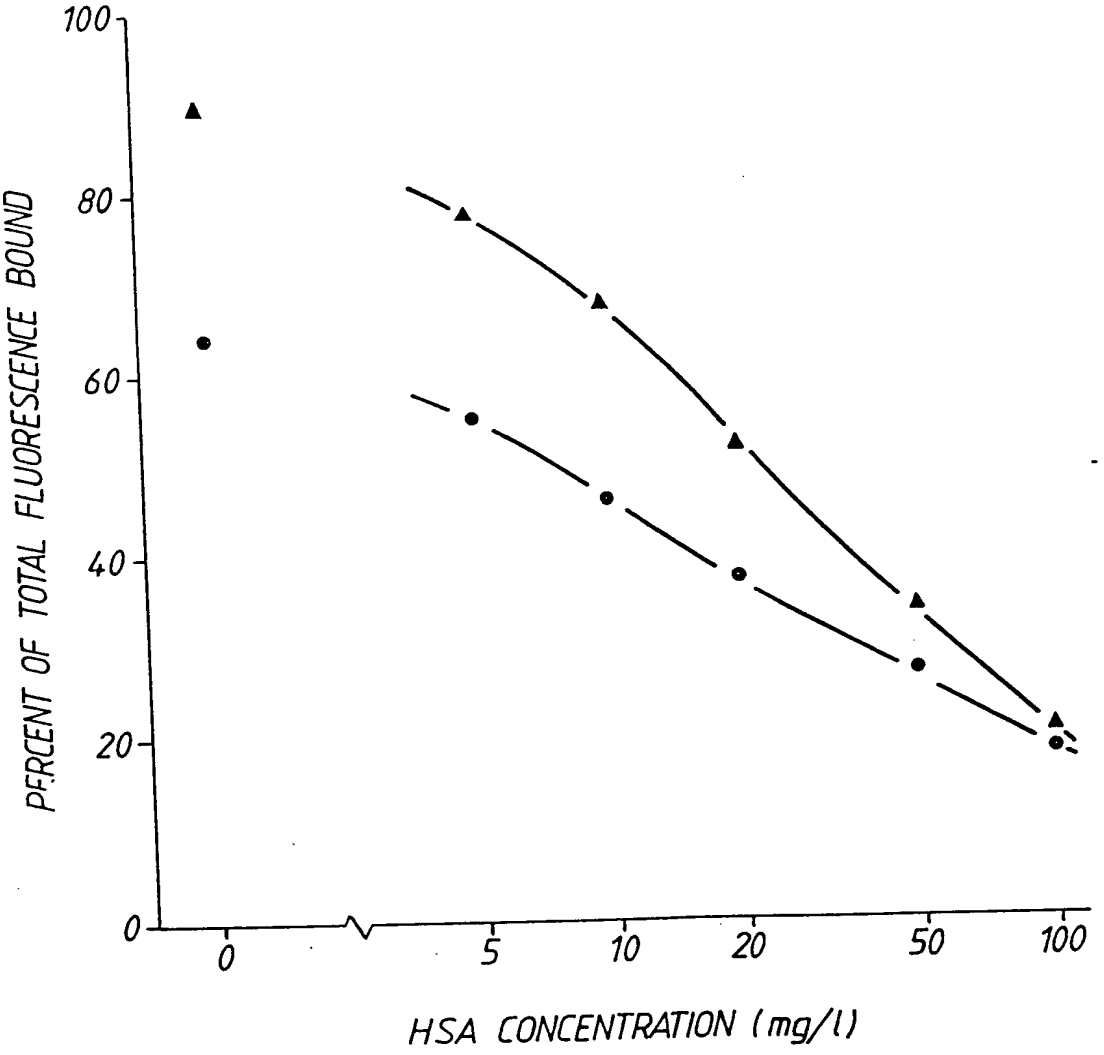
100
80
60
40
20
0
PERCENT OF TOTAL FLUORESCENCE BOUND
0
5
10
20
50
100
HSA CONCENTRATION (mg/l)

FIG. 1.

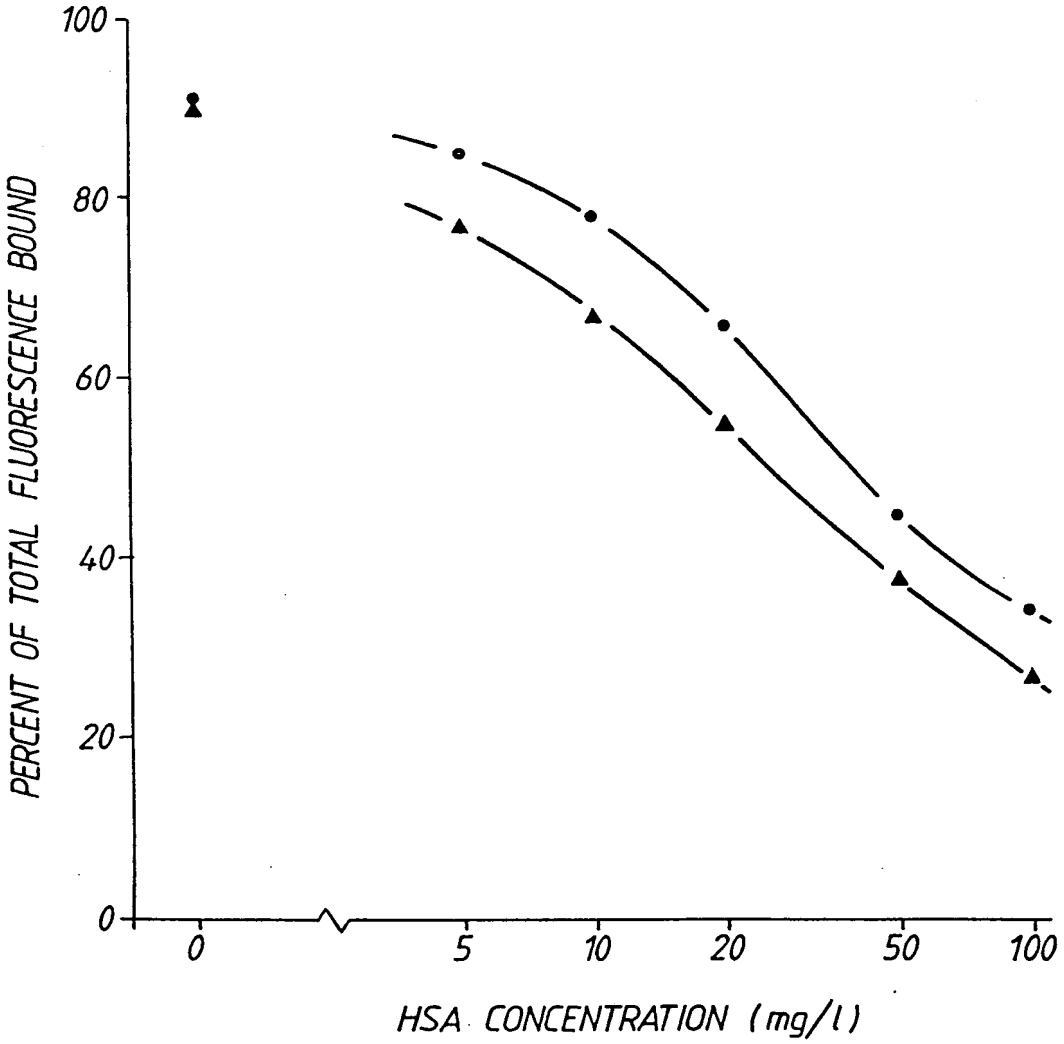
100
80
60
40
20
0
PERCENT OF TOTAL FLUORESCENCE BOUND
0
5
10
20
50
100
HSA CONCENTRATION (mg/l)

FIG. 2.

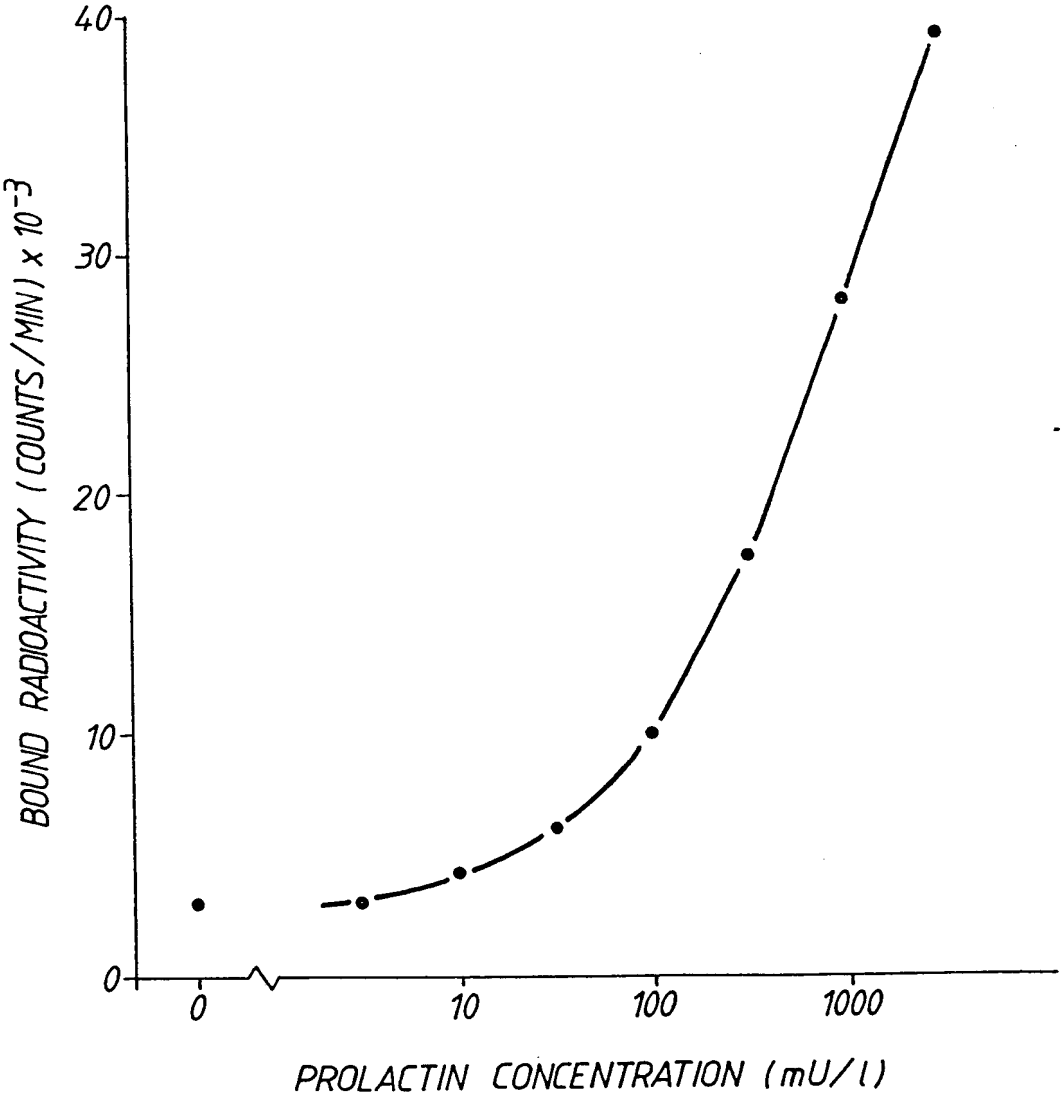
40
30
20
10
0
BOUND RADIOACTIVITY (COUNTS/MIN) x 10^{-3}
0
10
100
1000
PROLACTIN CONCENTRATION (mU/l)

FIG. 3.

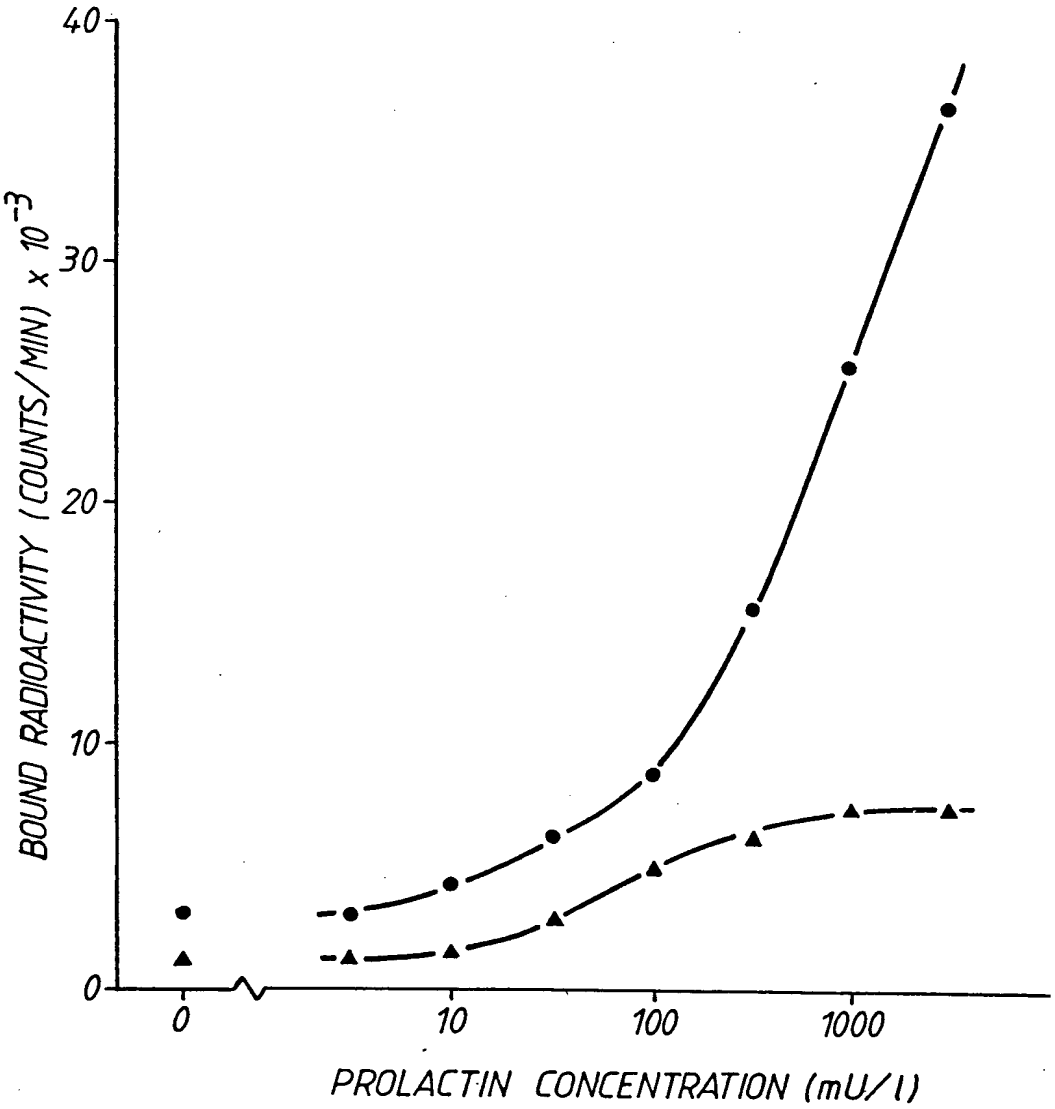
40
30
20
10
0
BOUND RADIOACTIVITY (COUNTS/MIN) x 10^{-3}
0
10
100
1000
PROLACTIN CONCENTRATION (mU/l)

FIG. 4.